DESCRIPTION

# CELL DEATH-INDUCING FUSION GENE SPECIFICALLY ACTING ON CANCER AND PRODUCT THEREOF

## Technical Field

The present invention relates to a protein having a strong cell death-inducing activity, that is, a fusion protein in which a modified Bax protein is fused with GFP at the N-terminus to construct a GFP-modified Bax protein and a homing signal peptide having a homing activity to a surface receptor of endothelial cells undergoing angiogenesis is further fused with the GFP-modified Bax protein at the N-terminus. The present invention also relates to a gene which codes for the protein and a drug which contains the protein to inhibit the growth of cancer cells.

## Background Art

Apoptosis is programmed cell death and the Bax gene is known to be a potent apoptosis-inducing gene. The Bcl-2 gene is known to be an oncogene which suppresses apoptosis and many Bcl-2 family proteins homologous to Bcl-2 protein are found (Bcl-2, Bcl-$x_L$ and the like).

Introducing genes that induce various types of cell death including apoptosis into cancer cells is a promising anti-cancer therapy. It has been reported recently that Bcl-2 family proteins such as Bcl-2, Bcl-$x_L$ and the like are expressed in cancer cells and exhibit antagonism to the induction of cell death by the Bax protein. These family proteins exhibit antagonism by binding to Bax protein via the region named BH3 of the Bax protein. The present inventors investigated an N-terminus deleted Bax (ΔNBax) which comprises an amino acid sequence from the 112th amino acid residue to the 192nd amino acid residue of Bax and has lost the N-terminal region including the BH3 region, and reported that ΔNBax is the cell death-inducing domain of the cell death-inducing *bax* gene product (Biochem Biophys Res Commun. 1998 Feb 13; 243 (2): 609-616). When located at the downstream of a promoter,

expression of the ΔNBax in cells induces cell death, which can not be inhibited by even overexpression of Bcl-$x_L$. The gene coding for ΔNBax, the vector containing the gene and the usage of the ΔNBax peptide for inhibiting the proliferation of cancer cells have been reported (JP Patent Publication (Kokai) No. 2002-355034).

On another front, various homing signal peptides are currently investigated to introduce drugs, such as anti-cancer drugs and the like, into cells specifically. For example, peptides designated as NGR and RGD are known to act selectively on the endothelial cells undergoing angiogenesis (Nat Med. 1999 Sep; 5(9):1032-1038), and it is possible to use these as specific homing signal peptides for the cell surface receptor of endothelial cells undergoing angiogenesis in cancer tissue.

Presently, cancer is treated mainly by chemotherapy in which anti-cancer drugs are administered, radiotherapy in which an affected lesion is irradiated, immunotherapy in which anti-cancer cell antibodies are administered and gene therapy. However, there are various problematic side effects in chemotherapy and radiation therapy. Also, immunotherapy requires a long period of time and further, gene therapy demands great efforts in developing because of the safety considerations such as uncertainty of the effect of the gene on patients and the like. Thus, cancer therapy in which a large-molecular-weight protein acting directly on cancer cells is directly targeted to the target site has been sought. However, there has been no protein which specifically acts on cancer cells to strongly inhibit the growth of cancer cells and can be surely used for treating cancer. If ΔNBax is specifically interacted with the surface receptor of the endothelial cells undergoing angiogenesis in cancer tissue, it is expected that ΔNBax is utilized as a more effective inhibitor of cancer cell growth, solving the shortcomings of the past treatment method. However, no investigation in this line has conventionally been carried out. ΔNBax itself possesses an apoptosis-inducing activity but its apoptosis-inducing activity has been desired to be enhanced.

## Disclosure of the Invention

The objective of the present invention is to enhance the apoptosis-inducing activity of ΔNBax, which is a modified Bax, and further to let ΔNBax act specifically on the cell surface

receptor of the endothelial cells undergoing angiogenesis. In particular, the objective of the present invention is to provide a fused protein, in which a homing signal peptide that is specific to the cell surface receptor of endothelial cells undergoing angiogenesis, Green Fluorescent Protein (GFP) and ΔNBax are fused in this order, and a gene coding for the fused protein and an anti-cancer drug consisting of the same.

The present inventors, to investigate the effect of ΔNBax, have constructed a fusion protein, in which GFP is fused to the N-terminus of ΔNBax to easily visualize the localization of ΔNBax in the cells, and introduced the fusion protein to established cell lines to investigate cell death-inducing activity. Surprisingly, it was discovered that the cell death-inducing activity was enhanced in ΔNBax fused with GFP. Furthermore, the present inventors, having investigated earnestly how GFP-fused ΔNBax with enhanced apoptosis-inducing activity is directed to specifically interact with the surface receptor of the endothelial cells undergoing angiogenesis in cancer tissue, have discovered that GFP-fused ΔNBax with enhanced apoptosis-inducing activity can specifically interacts with the surface receptor of the endothelial cells undergoing angiogenesis by fusing the homing signal peptide such as NGR, RGD and the like to the N-terminus of GFP-fused ΔNBax, completing the present invention.

Thus the present invention is as follows.

(1) A fusion gene comprising a cell death-inducing gene that acts specifically on a surface receptor of endothelial cells undergoing angiogenesis, wherein the fusion gene is produced by fusing, a gene that codes for a homing signal peptide sequence specific for the surface receptor of endothelial cells undergoing angiogenesis, a gene coding for green fluorescent protein (GFP) and a gene coding for ΔNBax protein, which is human Bax with a deletion of the N-terminal sequence including the BH3 region, in this order.

(2) The fusion gene according to (1), wherein the homing signal peptide sequence is selected from the group consisting of peptide sequences of (a) to (o) shown below:

(a) RGD peptide sequence,

(b) NGR peptide sequence,

(c) peptide sequence shown in SEQ ID NO: 7,

(d) peptide sequence shown in SEQ ID NO: 8,

(e) peptide sequence shown in SEQ ID NO: 9,

(f) peptide sequence shown in SEQ ID NO: 10,

(g) peptide sequence shown in SEQ ID NO: 11,

(h) peptide sequence shown in SEQ ID NO: 12,

(i) peptide sequence shown in SEQ ID NO: 13,

(j) peptide sequence shown in SEQ ID NO: 14,

(k) peptide sequence shown in SEQ ID NO: 15,

(l) peptide sequence shown in SEQ ID NO: 16,

(m) peptide sequence comprising LDV,

(n) peptide sequence shown in SEQ ID NO: 17 and

(o) peptide sequence shown in SEQ ID NO: 18.

(3) The fusion gene according to (2), wherein the homing signal peptide sequence is RGD or NGR which is a homing signal peptide specific for endothelial cells undergoing angiogenesis.

(4) The fusion gene according to any of (1)-(3), wherein ΔNBax protein, which is human Bax with a deletion of the N-terminal sequence containing the BH3 domain, comprises an amino acid sequence from the 112th to 192nd of the human BAX amino acid sequence.

(5) The fusion gene according to any of (1)-(3), wherein the fusion gene comprises following DNA of (p) or (q),

(p) a DNA having a nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 5.

(q) a DNA which hybridizes with a DNA having a complementary sequence of the DNA of (p) under a stringent condition and which codes for a protein which binds to endothelial cells undergoing angiogenesis and which has an enhanced cell death-inducing activity.

(6) An expression vector containing the fusion gene according to any of (1) to (5).

(7) The expression vector according to (6), which can express the fusion gene in a cell-free system.

(8) A method for producing the fusion protein encoded by any of the fusion gene according to (1) to (5), including a step of *in vitro* expression by the expression vector according to (7).

(9) A fusion protein containing a cell death-inducing protein that acts specifically on surface receptor of endothelial cells undergoing angiogenesis, wherein the fusion protein is generated by fusing, a homing signal peptide sequence specific for a surface receptor of endothelial cells undergoing angiogenesis, green fluorescent protein (GFP) and ΔNBax protein, which is human Bax with a deletion of the N-terminal sequence containing the BH3 domain, in this order.

(10) The fusion protein according to (9), wherein the homing signal peptide sequence is selected from the group consisting of peptide sequences of (a) to (o) shown below:

(a) RGD peptide sequence,

(b) NGR peptide sequence,

(c) peptide sequence shown in SEQ ID NO: 7,

(d) peptide sequence shown in SEQ ID NO: 8,

(e) peptide sequence shown in SEQ ID NO: 9,

(f) peptide sequence shown in SEQ ID NO: 10,

(g) peptide sequence shown in SEQ ID NO: 11,

(h) peptide sequence shown in SEQ ID NO: 12,

(i) peptide sequence shown in SEQ ID NO: 13,

(j) peptide sequence shown in SEQ ID NO: 14,

(k) peptide sequence shown in SEQ ID NO: 15,

(l) peptide sequence shown in SEQ ID NO: 16,

(m) peptide sequence comprising LDV,

(n) peptide sequence shown in SEQ ID NO: 17 and

(o) peptide sequence shown in SEQ ID NO: 18.

(11) The fusion protein according to (10), wherein the homing signal peptide sequence is RGD or NGR which is a homing signal peptide sequence specific for endothelial cells undergoing angiogenesis.

(12) The fusion protein according to (10) or (11), wherein ΔNBax protein, which is human Bax with a deletion of the N-terminal sequence containing the BH3 domain, consists of an amino acid sequence from the 112th to 192nd of the human BAX amino acid sequence.

(13) The fusion protein according to any of (10) to (12) shown in (r) or (s) below,

(r) a fusion protein having an amino acid sequence which is represented by SEQ ID NO: 4 or 6,

(s) a protein, which has the amino acid sequence of (r) in which one or some amino acids are deleted, substituted or added, and which binds to endothelial cells undergoing angiogenesis and has enhanced cell death-inducing activity.

(14) A cancer cell growth inhibitor containing the fusion protein according to any one of (10) to (13),

and

(15) The cancer cell growth inhibitor according to (14), wherein the cell death-inducing activity of ΔNBax protein, which is human Bax with a deletion of the N-terminal sequence containing the BH3 domain, is enhanced by fusion with green fluorescent protein (GFP) as compared with that of ΔNBax protein, which is human Bax with only the N-terminal sequence containing the BH3 domain deleted.

This application incorporates herein the content of the description of JP Patent Application No. 2003-081337 and/or drawings on which the priority of the present application is based.

## Brief Description of the Drawings

Figure 1 shows the result of the comparison of cell death-inducing activity of GFP-ΔNBax and ΔNBax;

Figure 2 is a photograph showing the incorporation of NGR-GFP-ΔNBax into a cell;

Figure 3 is a photograph showing an image of PI positive cells; and

Figure 4 shows anti-tumor effect of NGR-GFP-ΔNBax using a mouse bearing tumor.

## Best Mode for Carrying Out the Invention

The invention of this application is a fusion gene containing the cell death-inducing region of the cell death-inducing gene, human Bax, and a protein encoded by this gene, wherein the fusion gene contains a gene which codes for a homing signal peptide and a gene

which codes for GFP (green fluorescent protein) and is constructed by ligating the gene coding for the homing signal peptide, the GFP coding gene and the cell death-inducing region of Bax from the 5' end in this order.

Bax contains BH1, BH2 and BH3 domains, but the region responsible for the cell death-inducing activity of Bax is the area outside the BH3 domain. The Blc-2 family proteins, which antagonize the cell death-inducing activity of Bax protein, interacts with the BH3 domain. The core sequence of the BH3 domain is from the 59th to 73rd or 77th residue of the N-terminal amino acid sequence of Bax. The modified Bax (ΔNBax) is encoded by the gene which contains the cell death-inducing region and has the nucleotide sequence corresponding at least the N-terminal amino acid sequence from the 59th to 73rd or 77th amino acids deleted. As the gene coding for such a modified Bax containing the cell death-inducing region, this is preferably a polynucleotide of 243 bases which encodes the sequence of amino acid residues from residue 112 to residue 192 of the amino acid sequence of the Bax protein which consist of 192 amino acid residues and which is encoded by the Bax gene. The nucleotide sequence of the human Bax gene and the amino acid sequence of human Bax protein are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. Based on these sequence information, human Bax cDNA is synthesized by conventional genetic engineering technique and ΔNBax can be obtained using restriction enzymes and the like. For example, the method described in Biochem Biophys Res Commun. 1998 Feb 13: 243 (2): 609-616 can be used to obtain ΔNBax.

In the present invention, the ΔNBax gene is preferably a DNA which consists of a nucleotide sequence from nucleotide 334 to nucleotide 576 of SEQ ID NO: 1 (corresponding to residue 112 to residue 192 of the amino acid sequence of Bax protein) but also includes genes which hybridize with a DNA complementary to this DNA under a stringent condition and which also code for a protein having the cell death-inducing activity. The stringent conditions are, for example, that the sodium concentration is 500 to 1000 mM, preferably 700 mM, the temperature is 50 to 70°C, preferably 65°C. The ΔNBax gene includes DNA, which has homology to the nucleotide sequence from nucleotide 334 to nucleotide 576 of SEQ ID NO: 1 of at least 85% or above, preferably 90% or above, more preferably 95% or above and

especially preferably 97% or above, when calculated by BLAST (Basic Local Alignment Search Tool) or the like (for example, using the parameters of the initial or default setting) and also codes for a protein with the cell death-inducing activity. Further, the gene includes DNA in which one or more nucleotides in the nucleotide sequence from nucleotide 334 to nucleotide 576 of SEQ ID NO: 1 are deleted, substituted or added and which also codes for a protein having the cell death-inducing activity. The term "cell death-inducing activity" used herein means the activity of inducing cell death to cells, where cell death includes apoptosis and necrosis. For example, in one form of cell death, apoptosis, the characteristic morphological alterations occur such as chromosomal condensation in the cell nucleus, fragmentation of cell nucleus, loss of cell surface microvilli and condensation of cytoplasm. Originally, Bax has been found as a protein that induces apoptosis, but it has been reported that Bax causes necrosis in certain cell types (Shinomura, N., et al. Oncogene, Vol. 18:5703 (1999)). The term "cell death" used herein includes both of apoptosis and necrosis. It can be determined *in vitro* by the method according to Example 4 of the present description whether a particular protein has cell death-inducing activity or not.

GFP derived fromthe jellyfish *Aequorea victoria*, which is encoded by the *gfp10*gene of the jellyfish *Aequorea Victoria*, can be used (Prasher, D. C. et al. (1992), "Primary structure of the Aequorea victoria green fluorescent protein" Gene 111:229-233). Also, the commercially available GFP gene can be used. For example the gene coding for GFP included in a commercially available vector pGreenlantern (Invitrogene LifeTechnology) (JP Patent Publication (Kohyo) No. 2000-503536) can be used. The GFP-coding sequence is shown in SEQ ID NO: 3 from nucleotide 40 to nucleotide 753 (from nucleotide 28 to nucleotide 741 in SEQ ID NO: 5). Various modified types of GFP are also known and these modified types of GFP may be used as the GFP in the present invention. These modified types of GFP include EGFP (enhanced green fluorescent protein), GFPUV, GFPmut3.1, BFP2 (all are available from Clonetech Co.), Venus (Nature Biotechnology January 20002 Vol. 20-1, 87-90) and S65T. Mutant forms of the GFP protein that emit lights of different color, such as EBFP (blue), ECFP (Cyan) and EYFP (Yellow) (all available from Clonetech Co.) may also be used as GFP in the present invention. These modified types of GFP are described in detail in, for example,

"Experimental Course 3 in Post Genome Era, GFP and Bio-imaging, Supplement of Experimental Medicine" (Ed. by Miyawaki, Atsushi. Issued on October 25, 2000, from Yodosha Co., Tokyo) and can be obtained by referring to this article. Further, a GFP modified to enhance the cell death-inducing activity of ΔNBax when fused with ΔNBax, and its derivative protein are also included as GFP in the present invention.

A homing signal peptide is a peptide which binds to its cognate receptor(s) (ligand for the homing signal peptide) expressed on the surface of particular cells, and when it is administered into the body, it is circulated in the body fluid and binds to the target cells carrying the receptor on their surface. The homing signal peptide can bind specifically to the receptor of the target cells. When a potential drug protein or the like are bound to the C-terminus of the homing signal peptide, the homing signal peptide carries this protein to the target cells, and this protein is incorporated into the cells. In the invention of the present application, a peptide is used which specifically binds to endothelial cells undergoing angiogenesis by binding specifically to the surface receptor of endothelial cells undergoing angiogenesis. NGR and RGD, which specifically bind to the homing signal peptide receptor of endothelial cells undergoing angiogenesis (Nat Med. 1999 Sep; 5 (9): 1032-1038), can be used as the homing signal peptide and the nucleotide sequences of NGR and RGD are shown by the nucleotide sequences in SEQ ID NO: 3, from nucleotide 4 to nucleotide 33 and SEQ ID NO: 5, from nucleotide 7 to nucleotide 21, respectively. It is known that NGR binds to Aminopeptidase N (CD13) (Pasqualini, R., et al., Cancer Research (2000) 60: 722-727, and RGD binds to αvβ3 and αvβ5 of integrin (Koivunen, E., et al., Bio/Technology (1995) 13: 265-270). Since NGR or RGD, when used as the homing signal peptide, homes directly to endothelial cells undergoing angiogenesis, cell death-inducing protein fused with the homing signal peptide binds to the surface receptor of endothelial cells undergoing angiogenesis in cancer tissues, is incorporated into those endothelial cells and causes the death of cells undergoing angiogenesis in cancer tissue. Since cancer cells in cancer tissue are provided with nutrients needed for survival and growth from endothelial cells undergoing angiogenesis, the cancer cells die because they can not be provided with nutrients due to the death of these endotherial cells. Thus, cell death-inducing protein fused with the homing signal peptide has

killing effect on cancer cells at the final stage. Some cancer cells, after dedifferentiation, may express the receptor which is expressed on the surface of endothelial cells undergoing angiogenesis. In such cases, the homing signal peptide directly binds to the cancer cells, and the cell death-inducing protein is incorporated into the cancer cells causing the death of the cancer cells. It has been reported, for example, that NGR described above binds to KS1767 cancer cells derived from Kaposi sarcoma (Ellerby, H.M., et al. Nature medicine Vol. 5: 1032 (1999)), and therefore, the fusion protein with fused NGR of the present invention can cause directly the death of the cancer cells. However, the homing signal peptide is not limited to these peptides, but various peptides, which specifically bind to cells in particular tissues or organs, may be used for homing to cancer tissue of these tissue or organs. Examples of such peptides include followings.

(1) Organ specific homing signal peptides (Pasqualini, R. & Rouslahti, E. (Nature 1996 vol. 380, pp. 364-366.))

(a) The 4 signals, CLSSRLDAC (SEQ ID NO: 7), CNSRLHLRC (SEQ ID NO: 8), CENWWGDVC (SEQ ID NO: 9), WRCVLREGPAGGCAWFNRHRL (SEQ ID NO: 10) target the brain.

(b) The 2 signals CLPVASC (SEQ ID NO: 11), and CGAREMC (SEQ ID NO: 12) target the kidney.

(2) A homing signal peptide which targets synovial membrane (of the joints) (Lee, L. et al., Arthritis Rheum (2002) vol. 476, pp. 2109-2120.).

CKSTHDRLC (SEQ ID NO: 13)

(3) A homing signal peptide which targets tumor lymphatics (Laakkonen, P., et al., Nature Medicine (2002) vol. 8, pp. 751-755.).

CGNKRTRGC (SEQ ID NO: 14)

(4) A homing signal peptide which targets endothelial cells in blood vessels undergoing angiogenesis (Asai, T., et al., FEBS Letter (2002) vol. 520, pp. 167-170.).

APRPG (SEQ ID NO: 15)

(5) Peptides which bind to cell surface integrin (integrin is a collective name, and there are many different types of integrin) (Koivunen, E., et al., Method in Enzymology (1994) vol. 245, pp. 346-369.).

4 peptides, KQAGDV (SEQ ID NO: 16), LDV, KRLDGS (SEQ ID NO: 17), DGEA (SEQ ID NO: 18) are known.

Anti-cancer antigen antibodies which bind to cancer antigen or fragments of these antibodies may also be used in the present invention as a homing peptide because these antibodies and their fragments have a homing activity similar to that of the homing signal peptide.

The part of Bax containing its cell death-inducing region, GFP and the homing signal peptide are fused in the order so that the homing signal peptide is located at the N-terminus of the resultant protein, GFP is located next to the C-terminus of the homing signal peptide and the part of Bax containing its cell death-inducing region is located next to the C-terminus of GFP. This is designed because the homing signal peptide can bring protein that attached to the C-terminus of the homing signal peptide into the target cells and Bax contains also a membrane anchor region at the C-terminus. ΔNBax alone has the cell death-inducing activity and this activity is enhanced by fusing GFP to the N-terminus of ΔNBax. In the present invention, the enhanced cell death-inducing activity of the GFP-ΔNBax fusion protein means that its cell death-inducing activity is higher than that of ΔNBax alone. The higher activity of the cell death-inducing activity of the GFP-ΔNBax fusion protein can be determined by comparing the cell death-inducing activity of the GFP-ΔNBax with that of ΔNBax alone according to the method described in Example 1 (2) of the present description. For example, an appropriate expression vector containing the GFP-ΔNBax fusion gene or the ΔNBax alone is introduced into suitable host cells to express the GFP-ΔNBax fusion protein or ΔNBax protein alone. When the cell death-inducing activity is measured by the ratio of surviving cells, the cell death-inducing activity of the GFP-ΔNBax fusion protein is significantly stronger than that of ΔNBax alone, preferably 1.5 times stronger or above (the surviving cell ratio in the ΔNBax expression experiment is 1.5 times higher or above than that

inthe GFP-ΔNBax expression experiment), more preferably 2 times stronger or above, and especially 3 times stronger or above.

The fusion of the genes coding for the homing signal peptide, GFP and the cell death-inducing region of ΔNBax can be carried out by conventional genetic engineering techniques. At this time, appropriate restriction enzyme sites may be introduced and used to construct the fusion gene. As described above, the fusion is carried out in the order of: the gene coding for the homing signal peptide; the gene coding for GFP; and the gene coding for the cell death-inducing region of Bax. Here, care should be taken so that no stop codon emerges in the fused genes. The distance between the fused gene is not limited, and a linker sequence may be placed between them. The 3 genes should be fused in-frame in order that the fusion protein is translated to exhibit the homing activity and the enhanced cell death-inducing activity. SEQ ID NO: 3 shows the nucleotide sequence of the gene coding for the fusion protein which contains RGD as the homing signal peptide and ΔNBax consisting of an amino acid sequence from residue 112 to residue 192 of human Bax protein. SEQ ID NO: 5 shows the nucleotide sequence of the gene coding for the fusion protein which contains NGR as the homing signal peptide and ΔNBax consisting of the amino acid sequence from residue 112 to residue 192 of human Bax protein. Genes containing a DNA sequence which hybridize with the sequence complementary to the DNA sequence described above under stringent conditions, and whose products bind to endothelial cells undergoing angiogenesis and demonstrate stronger cell death-inducing activity than ΔNBax alone, are included in the present invention. The stringent conditions are, for example, that the sodium concentration is 500-1000 mM, preferably 700 mM, the temperature is 50-70°C, preferably 65°C. Also included in the present invention are genes containing DNA, which has homology to of the nucleotide sequence shown in SEQ ID NO: 3 or 5 of at least 85% or above, preferably 90% or above, more preferably 95% or above and especially preferably 97% or above when calculated by BLAST or the like (for example, using the parameters of the initial setting which is the default setting), which codes for a fusion protein which binds to endothelial cell undergoing angiogenesis and which has stronger cell death-inducing activity than ΔNBax alone. Further, included in the present invention is genes containing DNA in which one or more of the

nucleotides in the nucleotide sequence of SEQ ID NO: 1 are deleted, substituted or added and which also codes for a protein which binds to endothelial cells undergoing angiogenesis and which has stronger cell death-inducing activity than ΔNBax alone.

In cases where homing signal peptides other than RGD and NGR are used, the fusion gene of the present invention includes a fusion gene which has a sequence with deletion, substitution or addition of some nucleotides as mentioned above, as long as a resultant fusion protein binds to target cells to exhibit the enhanced cell death-inducing activity.

The fusion gene constructed as described above can be cloned into an available appropriate expression vector to express, recover and purify the fusion. Here, it is preferable to express the fusion protein in a cell-free expression system because, when the expression vector is introduced into host cells to express the fusion protein, the host cells easily die due to the action of cell death-inducing activity of ΔNBax. The cell-free expression system herein means to express the gene product in the expression vector containing the gene *in vitro* by mixing the vector with required reagents in a appropriate container, without introducing the vector to host cells (Spirin, A.S. et al., (1988) "A continuous cell-free translation system capable of production polypeptides in high yield" Science 242, 1162; Kim, D.M., et al., (1996) "A highly efficient cell-free protein synthesis system from E. coli" Eur. J. Biochem. 239, 881-886). Protein can be expressed using a commercially available cell-free expression kit. Such kits include, for example, Rapid Translation System (RTS) (Roche) and Expressway In Vitro Protein Synthesis System (Invitrogen) and the like. In using these kits, the expression vector to be used is not limited, but there are suitable vectors for each cell free expression kit and these may be used. The expression vector for the former kit includes pIVEX2.2bNde, and the expression vector for the latter kit includes pEXP1 and pEXP2.

In *in vivo* expression system, where the expression vector containing the fusion gene of the present invention is introduced into host cells to express the fusion protein of the present invention, the host cells can not grow due to cell death by the effect of cell death-inducing protein when the fusion gene is constitutively expressed. Therefore, it is necessary to use host cells suitable for an inducible gene expression system so that induction of the fusion gene expresseion is controlled to allow the expression during the period from just after the host cells

sufficiently grow until cell death is caused by the expressed fusion protein. By using the host cells suitable for the inducible expression system, induction of the expression, when the host cells containing the fusion gene grow to reach a sufficient number of cells, allows to obtain a sufficient amount of fusion protein until cell death of the host cells occurs. A vector capable of inducible expression of the gene is the vector in which the expression of the inserted exogenous gene is induced by a particular treatment. For example, an inducible expression vector can be constructed by introducing a promoter that is inducible or repressible by a particular regulatory agent or temperature condition into a vector. Some promoters are known to be inducible in a inducer-specific manner, when an inducer is added to the culture medium of host cells. For example, the *lac* promoter and the *tac* promoter is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). IPTG is also used as an inducer for T7 RNA polymerase in the T7 promoter system when T7 RNA polymerase DNA(T7 DNA1) is linked to the downstream of the *lac* promoter. Further, expression of the *trp* promoter is inducible by 3β-indolyl acrylic acid. Gene expression can be induced not only by addition of an inducer but also by changing the temperature of the culture. The recombinant host cells which express λcIts repressor and carry an expression vector containing $\lambda P_L$-promoter is cultured, for example, at about 15-36°C, preferably about 30-36°C and then the induction of gene expression is preferably carried out by inactivating λcIts repressor at about 37-42°C. When T7 RNA polymerase DNA (T7 DNA1) in the T7 promoter system is linked to the downstream of the $\lambda P_L$-promoter, the T7 promoter is activated by binding of T7 phage RNA polymerase 1 generated by raising the culture temperature.

Further, cells resistant to the cell death-inducing protein may be used as host cells. For example, *Escherichia coli* (*E. coli*) cells and the like that are resistant to ΔNBax may be used. Such resistant bacteria can be obtained by inducing mutation by chemical mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine and the like or physical mutagens such as ultraviolet light and the like and screening for the bacteria resistant to the cell-death-inducing protein. For example, when the recombinant *E. coli* cell carrrying the fusion gene of the present invention is artificially mutagenized, the bacterial cells that can grow are mutant *E. coli* cells resistant to ΔNBax. After introduction of a gene encoding ΔNBax (or the fusion

gene of the present invention) into *E. coli* cells which are artificially mutagenized, the cells which can grow are mutant *E. coli* cells resistant to ΔNBax. Chemical and physical mutagens which cause mutation in genes, and the method of usage and dose of the mutagens are known to those skilled in the art, and the method for screening for the resistant cells may be designed appropriately by those skilled in the art.

Expression of the fusion protein by introducing the expression vector containing the fusion gene according to the present invention into host cells is carried out as follows.

Any vector derived from plasmid, phage, virus and the like, which are capable of replication in host cells, may be used. Vectors include, for example, *E. coli* plasmids, such as pBR322, pBR325, pUC118, pUC119, pKC30, pCFM536 and the like, *Bacillus subtilis* plasmids, such as pUB110 and the like, yeast plasmids, such as pG-1, YEp13, YCp50 and the like, phage vectors, such as λgt110, λZAPII and the like. Expression vectors for mammalian cells include virus DNA such as baculovirus, vaccinia virus, adeno virus and the like, SV40 and its derivatives. The vectors may contain an origin of replication, selection marker and promoter, and if necessary, an enhancer, a terminator, a ribosomal binding site, a polyadenylation signal and the like. As described above, an inducible promoter should be used to induce the expression of the inserted gene.

The vectors which can be used include commercially available ones, for example, bacterial vectors such as pEF1, pPROEX (Invitrogen), pQE30, pQE31, pQE32, pQE70, pQE60, pQE-9 (Qiagen), pGEX-5X-1, pGEX-5X-2, pGEX-5x-3, pBluescriptII KS, ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia), pET3a, pET3b, pET3c, pET-11a (Novagene), pUC118 (Takara), and eukaryotic vectors such as pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVL, SV40 (Pharmcia) and the like.

The replication origins which can be used for the *E. coli* vectors include, for example, those of Col E1, R factor and F factor, those for the yeast vectors include, for example, those of 2μm DNA and ARSI, and those for the mammalian vectors include, for example, those of SV40, adenovirus and bovine papilloma virus. The promoters which can be used include adenovirus or SV40 promoter, *E. coli lac* or *trp* promoter, phage lambda $P_L$ promoter , ADH,

PH05, GPD, PGK and AOX1 promoters for yeast, a promoter derived from nucleopolyhedrovirus for silkworm cells and the like.

The selection markers which can be used include the kanamycin resistant gene, ampicilline resistant gene, tetracycline resistant gene and the like for *E. coli* vectors, the Leu2, Trp1, Ura3 and the like genes for yeast vectors, and the neomycin resistant gene, hygromycin resistant gene, thymidine kinase gene, dihydrofolate reductase gene and the like for mammalian cells. For inducible expression of the fusion as described above, a promoter capable of inducing the expression of downstream genes should be used.

DNA can be cloned into a vector by any method. The vector preferably contains either a poly-linker in which various restriction enzyme sites are present or a single restriction enzyme site. A particular restriction site in the vector can be cleaved with a restriction enzyme and a DNA can be inserted into the cleaved site. The expression vector containing the fusion gene of the present invention can be introduced into appropriate host cells to express and produce the protein encoded by the fusion gene described above.

The host cells include *E. coli* cells such as HB101, DH5, TG1, JM109, XL1-blue, BL21 (DE3), BL21 (DE3) pLysS and the like, bacterial cells such as and *Bacillus subtilis* and the like, fungal cells such as *Streptomyces, Aspergillus* and the like, yeast cells such as baker's yeast, methylotrophic yeast and the like, insects cells such as *Drosophila* S2, *Spodoptera* Sf9 and the like, mammalian cells such as CHO, COS, BHK, 3T3, C127 and the like. As described above, the cells resistant to the cell death-inducing protein included in the cell death-inducing fusion protein of the present invention, can also be used as a host.

Introduction of DNA into a host cell can be carried out by the conventional method using calcium chloride, calcium phosphate or DEAE dextran, electroporation and the like.

The recombinant fusion protein thus produced in host cells can be purified by various protein purification methods. For example, ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, affinity chromatography and the like may be used singly or in appropriate combinations. When the product is expressed as a fusion protein with GST and the like, the product can be purified based on the nature of the fusion protein or the peptide fused to the target protein. For example, a protein expressed as a fusion protein with an

amino acid sequence containing 6 consecutive histidine residues or more (i.e. histidine tag) can be purified using a chelate column because protein having histidine tag binds to the chelate column. A protein expressed as a fusion protein with GST can be purified efficiently using an affinity column in which glutathione is coupled to a carrier because GST has an affinity to glutathione.

The product thus produced is a fusion protein of a homing signal peptide, GFP and ΔNBax in this order from the N-terminus. The amino acid sequence of the fusion protein, in which the homing signal peptide is RGD and ΔNBax is the peptide from the residue 112 to residue 192 of human Bax protein, is shown in SEQ ID NO: 4. SEQ ID NO: 6 shows the amino acid sequence of the fusion protein in which the homing signal peptide is NGR and ΔNBax is the peptide from residue 112 to residue 192 of human Bax protein.

The fusion proteins in the present invention may have mutations such as deletion, substitution or addition of at least 1, preferably one or a few amino acids in these amino acid sequences, as long as they bind to endothelial cells undergoing angiogenesis and have stronger cell death-inducing activity than that of ΔNBax alone.

For example, at least one, preferably one or a few (for example, 1 to 10, more preferably 1 to 5) amino acids in the amino acid sequence shown in SEQ ID NO: 4 or SEQ ID NO: 6 may be deleted, or at least one, preferably one or a few (for example, 1 to 10, more preferably 1 to 5) amino acids in the amino acid sequence shown in SEQ ID NO: 4 or SEQ ID NO: 6 may be added, or at least one, preferably one or a few (for example, 1 to 10, more preferably 1 to 5) amino acids in the amino acid sequence shown in SEQ ID NO: 4 or SEQ ID NO: 6 may be substituted with other amino acids. The deletion, addition and substitution of amino acids may occur in any part of the homing signal peptide, GFP, or ΔNBax protein of the fusion protein.

Also included in the present invention is proteins which have homology to the amino acid sequence described above of at least 85% or above, preferably 90% or above, more preferably 95% or above and especially preferably 97% or above when calculated by BLAST or the like (for example, using the parameters of the initial setting which is a default setting).

A fusion protein using a homing signal peptide other than RGD and NGR may be included as the fusion protein of the present invention, even with deletion, addition and substitution in part of the amino acid sequence as described above, as long as its homing signal peptide binds to the target cells and it has enhanced cell death-inducing activity.

The present invention also includes a composition for cancer cell growth inhibitor containing the fusion protein described above as an active ingredient. This composition can be administered in various forms. The administration forms include oral formulation such as tablets, capsules, granules, powder, syrup and the like, or non-oral formulation such as injections, drip infusions, suppositories and the like. The cancer cell growth inhibitor of the present invention may also be administered to the cancer tissue directly. This cancer cell growth inhibitor is produced by known art and contains a carrier, diluent and excipient , which are normally used in pharmaceutical formulations. For example, lactose, magnesium stearate and the like are used as the carrier and excipient for tablets. The injections are prepared by dissolving, suspending or emulsifying the fusion protein in a sterile aqueous or oil based liquid normally used for injection. The aqueous solution for injection includes physiological saline, isotonic solution containing glucose or other supplements, and the like, and an appropriate solubilizer, for example, alcohol, polyalcohol such as propylene glycol, and non-ionic detergents may be used in combination. Oil based liquid includes sesame oil and soybean oil and the like, and benzylbenzoic acid, benzyl alcohol and the like as a solubilizer may be used in combination. Since the dosage is dependent of the symptoms, age, body weight and administration rout, it should be determined according to the judgment of the attending physician and the condition of the patient. Effective dosage may be obtained through *in vitro* tests or in vivo animal model test systems. For example, in mice bearing tumor, the volume of tumor is decreased by administering the fusion protein of the present invention at 50 μl of 500 ng/μl solution twice directly to the tumor of 0.2-0.4 $cm^3$ size.

The present invention is explained concretely by following examples but the present invention is not limited by these examples.

[Example 1] Construction of GFP-ΔNBax.

(1) Construction of GFP-ΔNBax

The GFP (Green Fluorescent Protein) gene (DNA fragment A) and the ΔNBax gene (DNA fragment B) were linked by the two-step PCR method.

DNA fragment A was amplified using a 5'-end primer (Primer 1) and 3'-end primer (Primer 2) and pGreenlantern (Invitrogen LifeTechnology) as a template. The 3'-half of Primer 1 contains the nucleotide sequence of the sense strand from the initiation codon of the GFP gene, and there is a restriction site (ATCGAT) of restriction enzyme ClaI at the 5'-end of the primer. The 5'-half of Primer 2 contains the anti-sense sequence of the 5'-end of the ΔNBax gene (from Ala 112 to Ser 118) and is complementary to Primer 3. Also, the 3'-half of the primer has an anti-sense nucleotide sequence of the 3'-end of the GFP gene except the termination codon.

DNA fragment B was amplified using a combination of Primer 3 and Primer 4 and pEF1BOS-Bax (Biochem Biophys Res Commun. 1998 Feb 13; 243 (2):609-616) as a template. The 5'-end primer, Primer 3, is the sense nucleotide sequence of the 5'-end of the ΔNBax gene (coding for amino acid residue from Ala 112 to Ser 118 of Bax). The 3'-end primer, Primer 4, is the anti-sense sequence of the 3'-endof the ΔNBax gene (the 3'-end of Bax) containing the termination codon, and there is a restriction site (TCTAGA) of restriction enzyme Xba I at the 5'-end of the primer.

Detail of the PCR reaction is as follows.

Reaction mixture (volume 100 μl): 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dCTP, dTTP, dGTP

AmpliTaqGold: 2.5 U

Pair of primers: a combination of Primer 1 and Primer 2, or a combination or Primer 3 and Primer 4 (each primer 1 μM)

Template DNA: 100 ng

Reaction condition 1: 94°C/10 min; (94°C /30 sec; 54°C /30 sec; 72°C /1 min) × 15 cycles; 72°C /3 min

After the reaction, the two amplified DNA fragments (A, B) were purified by 5% polyacrylamide gel electrophoresis. Next, the DNA fragments A and B (each 50 ng) were combined and mixed with the PCR reaction mixture described above (25 μl) to synthesize

each complementary strand using AmpliTaqGold. The condition of the synthesis was following Reaction condition 2.

Reaction condition 2: 94°C/10 min; (94°C/30 sec; 36°C-42°C/30 sec; 72°C/1 min) × 5 cycles; 72°C/3 min

After the reaction, 75 μl of the PCR reaction mixture containing Primer 1 and Primer 4 (final concentration 1 μM each), and AmpliTaqGold (2.5 U) was added, and the PCR reaction was carried out by following Reaction condition 3.

Reaction condition 3: 94°C/10 min; (94°C/30 sec; 54°C/30 sec; 72°C/1 min) × 12 cycles; 72°C/3 min

The 960-bp PCR product was purified by 5% polyacrylamide gel electrophoresis and cleaved with restriction enzymes, ClaI and XbaI. The fragment was cloned at the ClaI and XbaI restriction sites of the mammalian cell expression vector pEF-LACAB to obtain pEF-LACAB/GFP-ΔNBax.

(2) Comparison of cell death-inducing activity between GFP-ΔNBax and ΔNBax.

The vector pEF-LAC was used to express ΔNBax and GFP-ΔNBax in mammalian cells (Edamatsu, H., Kaziro, Y., Itoh, H. Inducible high-level expression vector for mammalian cells, pEF-LAC carrying human elongation factor 1 alpha promoter and *lac* operator. Gene (1977) 187: 289-294). A DNA fragment, in which the initiation codon ATG was attached to the 5'-end of the nucleotide sequence coding ΔNBax (from nucleotide 754 to nucleotide 999of SEQ ID NO: 3), was inserted into the multiple cloning sites (the XbaI restriction site) located at the downstream of the EF1α promoter of pEF-LAC to construct pEF-LAC-ΔNBax. As a control, plasmid pEF-LAC was used.

Similarly, another DNA fragment which contained the nucleotide sequence coding GFP-ΔNBax (from nucleotide 40 to nucleotide 999 of SEQ ID NO: 3) was cloned into the multiple cloning sites (between the ClaI restriction site and the XbaI restriction site) located at the downstream of the EF1α promoter of pEF-LAC to construct pEF-LAC-GFPΔNBax.

The cell death-inducing activity was measured by introducing the plasmid DNA described above into Jurkat cells to count by flow cytometry the number of surviving cells in which the gene was introduced. The number of surviving cells transfected with the plasmid

DNA described above was compared with that of cells in which the control plasmid is introduced.

Jurkat cells were co-transfected with pEF-LAC-ΔNBax 2 μg and GFP expression plasmid, pGreenLantern (Invitrogen Life Technologies), 1 μg using SuperFect transfection kit (Qiagen). The method described in the manual of the kit was used. As a control, the mixture solution of pEF-LAC (empty vector) 2μg and pGreenLantern 1 μg was similarly co-transfected into Jurkat cells. Since added amount of pGreenLantern is less than that of pEF-LAC-ΔNBax or pEF-LAC , the cells that pGreenLantern is introduced into, should be transfected with pEF-LAC-ΔNBax or pEF-LAC. Jurkat cells were transfected with 2 μg of pEF-LAC-GFPΔNBax using SuperFect transfection kit by the similar method described above. The living transfected cells emit green fluorescent light.

After the transfection, cells were cultured in RPMI1640 medium (Invitrogen Life Technologies) containing 10% FBS in 5% $CO_2$/95% Air, at 37°C (BIO-LABO Juji Field Inc.) for 2 days and analyzed by flow cytometry (COULTER Co. EPICS ELITE ESP). Fifty thousand cells of normal size were selected (gated) by forward scattering (FS) and side scattering (SS), among which the number of cells emitting green fluorescent light (Em. 488 nm) of GFP was counted.

Result is shown in Figure 1, in which the number of living cells in control pEF-LAC is given as 100. The number of living cells transfected with the GFPΔNBax gene was markedly smaller than that of living cells transfected with the ΔNBax gene, confirming that the cell death-inducing activity of GFPΔNBax is enhanced.

[Example 2] Construction of RGD-GFP-ΔNBax and NGR-GFP-ΔNBax.

To link a homing signal sequence specific to endothelial cells (RGD and NGR) to the 5'-end of the GFP-ΔNBax fusion gene, an *E. coli* expression vector, pPROEX1 (Invitrogen Life Technology), was used. The vector pEF-LACAB and the GFP gene (nucleotide 166) in the GFP-ΔNBax fusion gene have 3 and 1 NcoI restriction sites, respectively. pEF-LACAB/GFP-ΔNBax was cleaved into 4 DNA fragments by NcoI, and the DNA fragment with about 1.3 kb long, which contains the 794-bp-long 3'-region (NcoI-XbaI) of the GFP-ΔNBax fusion gene (total length 980 bp), was cloned at the NcoI site of pPROEX1 in the

right orientation to obtain pPROEX1/ΔNGFP-ΔNBax. Two NotI restriction sites are present in the sequence derived from pEF-LACAB (7 bp downstream of the XbaI site) and in the sequence of the vector pROEX1 at the downstream of 3'-end of cloned fragment (the NcoI site at the 3'-end). pPROEX1/ΔNGFP-ΔNBax is cleaved with Not I to remove the sequence derived from pEF-LACAB including the NcoI site at the 3'-end. The resultant plasmidpPROEX1/ΔNGFP-ΔNBax/ΔNotI was obtained.

Each homing signal peptide, RGD and NGR, was linked to the N-terminus of ΔNGFP-ΔNBax by the PCR method. Primer 5 contains the NcoI restriction site at the 5'-end, the nucleotide sequence coding for the homing signal peptide RGD and the nucleotide sequence of the 5'-end of the GFP gene at the 3'-end. Primer 5 and pEF-LACAB/GFP-ΔNBax (each 20 ng) were mixed with the PCR reaction mixture (25 μl) described above, and the complementary strand of Primer 5 was synthesized using AmpliTaqGold. The condition for the synthesis was the reaction condition 4 described below.

Reaction condition 4: 94°C/10 min; (94°C/30 sec; 44°C-50°C/30 sec; 72°C/1 min) × 6 cycles.

After the reaction, 75 μl of the PCR reaction mixture containing Primer 6 and Primer 7 (final concentration 1 μM each) and AmpliTaqGold (2.5 U) was added and PCR was carried out under the condition 5 described below.

Reaction condition 5: 94°C/10 min; (94°C/30 sec; 50°C/30 sec; 72°C/1 min) × 16 cycles.

Primer 6 contains the nucleotide sequence of the 5'-half of Primer 5, and Primer 7 has the anti-sense nucleotide sequence of the GFP gene from nucleotide 200 to nucleotide 217. The PCR product was purified by 5% polyacrylamide gel electrophoresis and cleaved with restriction enzyme NcoI. This fragment was cloned to NcoI-cleaved pPROEX1/ΔNGFP-ΔNBax/ΔNotI to obtain pPROEX1/RGD-GFP-ΔNBax/ΔNotI. From this plasmid, GFP-ΔNBax carrying the homing signal peptide RGD at the N-terminus, RGD-GFP-ΔNBax, is produced. The nucleotide sequence was confirmed by DNA sequencing.

Primer 8 contains the NcoI restriction site at the 5'-end, the nucleotide sequence coding the homing signal peptide NGR and the 5'-end of the GFP gene at the 3'-end. The complementary strand of Primer 8 was synthesized by mixing Primer 8 and pEF-LACAB/GFP-ΔNBax (20 ng each) with the PCR reaction mixture described above and using AmpliTaqGold. The synthetic condition was the same as the reaction condition 4. After the reaction, 75 μl of the PCR reaction mixture containing Primer 9 and Primer 7 (final concentration 1 μM each) and AmpliTaqGold (2.5 U) was added and PCR was carried out under the reaction condition 5 described above. Primer 9 has a nucleotide sequence of the 5'-halfof Primer 8. The PCR product was purified by 5% polyacrylamide gel electrophoresis and cleaved with restriction enzyme NcoI. This fragment was cloned to NcoI-cleaved pPROEX1/ΔNGFP-ΔNBax/ΔNotI to obtain pPROEX1/NGR-GFP-ΔNBaΔx/NotI. From this plasmid, GFP-ΔNBax carrying the homing signal peptide NGR at the N-terminus, NGR-GFP-ΔNBax, is produced. The nucleotide sequence was confirmed by DNA sequencing.

[Example 3] Subcloning of the RGD-GFP-ΔNBax gene and the NGR-GFP-ΔNBax gene into pIVEX2.2bNde and production of these gene products in a cell free protein synthesis system.

To produce NGR-GFP-ΔNBax and RGD-GFP-ΔNBax in a cell free protein synthesis system, these genes were subcloned to a plasmid vector pIVEX2. 2bNde (Roche). The method for subcloning of the genes consisted of 2 steps using NcoI sites in the GFP gene as in Example 2.

pPROEX1/NGR-GFP-ΔNBax/NotI was cleaved with NcoI and XhoI, and a 822-bp DNA fragment containing the 794bp-long 3'-region of the GFP-ΔNBax fusion gene (total length 960 bp) was recovered. This DNA fragment was cloned to the vector pIVEX2.2bNde cleaved with NcoI and XhoI beforehand to obtain pIVEX2. 2bNde/ΔNGFP-ΔNBax.

As in Example 2, the 206-bp NcoI DNA fragments of the NGR-GFP and RGD-GFP genes were amplified by the PCR method using pPROEX1/NGR-GFP-ΔNBax/ΔNotI and pPROEX1/RGD-GFP-ΔNBax/ΔNotI as templates, respectively. After recovering the DNA fragments, they were subcloned to NcoI-treated pIVEX2. 2bNde/ΔNGFP-ΔNBax to obtain pIVEX2.2bNde/NGR-GFP-ΔNBax and pIVEX2.2bNde/RGD-GFP-ΔNBax. By adding these

plasmid DNAs to the RTS500HY kit reagent (Roche) according to the manual of Roche, RGD-GFP-ΔNBax protein and NGR-GFP-ΔNBax protein were synthesized using a cell system protein synthesis instrument, RTS Proteomaster (Roche). The synthesized proteins were confirmed and assayed(bovine serum albumin was used as the standard) by silver staining method using 2D Silver Staining Solution II (Daiichi Pure Chemicals Co.) and by conventional Coomassie Brilliant Blue staining method after SDS-PAGE electrophoresis using a PAG Mini gel (Daiichi Pure Chemicals Co.) stained.

Primer 1. 5' –NNATCGATCCACCATGAGCAAGGGCGAG -3' (SEQ ID NO: 19)

Primer 2. 5' –CTGGCAAAGTAGAAAAGGGCCTTGTACAGCTCGTC -3' (SEQ ID NO: 20)

Primer 3. 5' –GCCCTTTTCTACTTTGCCAG -3' (SEQ ID NO: 21)

Primer 4. 5' –NNTCTAGATCAGCCCATCTTCTTCCA -3' (SEQ ID NO: 22)

Primer 5. 5' –CCATGGCCTGCGATTGCCGTGGTGATTGTTTTTGTGGTGG TATGAGCAAGGGCGAGG -3' (SEQ ID NO: 23)

Primer 6. 5' –NNNNCCATGGCCTGCGATTGCC -3' (SEQ ID NO: 24)

Primer 7. 5' –TGGAAAAGCACTGCACGC -3' (SEQ ID NO: 25)

Primer 8. 5' –CCATGGCCTGCAACGGTCGTTGCGGTGGTATGAGCAAGG GCGAGG -3' (SEQ ID NO: 26)

Primer 9. 5' –NNNNCCATGGCCTGCAACGGTC -3' (SEQ ID NO: 27)

[Example 4]

(1) Cell culture.

HUVEC (human umbilical vein endothelial cell: Sanko Junyaku Co.) cells were used as cells undergoing angiogenesis and HeLa cells were used as control cells. EBM-2 (Sanko Junyaku Co.) and its supplement factors kit (including serum, antibiotics. Sanko Junyaku Co.) were used as the medium for HUVEC. HeLa cells were cultured in DMEM/F12 (Invitrogen LifeTechnology) supplemented with 10% FBS (fetal bovine serum; Sanko Junyaku Co.) and 1% penicillin-streptomycin (LifeTechnology).

(2) Introduction of protein and measurement of cell death-inducing activity.

HUVEC cells ($1.0 \times 10^3$ cells/well) and HeLa cells ($5.0 \times 10^2$ cells/well) were plated in a 96 well plate. Two hundred μl of medium per well was added. NGR-GFP-ΔNBax was produced by RTS500HY kit (Roche). The synthetic reaction mixture 20 μl was centrifuged (12,000 rpm, 4°C, 10 minutes). After removing the supernatant, the precipitant was re-dissolved in 20 μl of a dissolving solution (6M UREA, 0.15M NaCl, 20 mM Hepes pH7.2). After standing at room temperature for 10 min, the solution was centrifuged (12,000 rpm, 4°C, 10 minutes), and the supernatant was used as the sample of NGR-GFP-ΔNBax. The concentration of NGR-GFP-ΔNBax protein was determined to be 150 ng/μl by staining with coomassie brilliant blue (CBB) after conventional SDS-PAGE, using bovine serum albumin of known concentration as the standard. One hundred and fifty μl of the cell culture medium removed from the well was mixed with NGR-GFT-ΔNBax, the amount of which is shown below, and then returned to the well. After 24 and 48 hours of the addition of the protein, PI and Hoechest 33342 were added to the medium at 5 μM to determine the cytotoxicity. The numbers of PI positive cells and Hoechest positive cells were counted under a fluorescent microscope (LEICA DMIRB). A total of 1000 cells were counted in each well in 6 fields (100 × field) not overlapping each other.

Two experiments were carried out. In Experiment 1, only HUVEC cells were used. HUVEC cells were treated with NGR- GFP-ΔNBax (750 ng), or treated with the same volume (5 μl) of the dissolving solution (6M UREA, 0.15 M NaCl, 20 mM Hepes pH 7.2) as control. Results were evaluated after 48 hours. In Experiment 2, HUVEC cells and HeLa cells were used and NGR-GFP-ΔNBax (200 ng and 60 ng) was added to both cells, and results were evaluated after 24 hours. Table 1 shows the results. As shown in Table 1, cell death (PI positive rate) after 48 hours occurred more frequently in HUVEC cells treated with NGR-GFP-ΔNBax fusion protein than in the control to which only the solvent of an equal volume was added, where HUVEC cells are the model of endothelial cells undergoing angiogenesis. Also, when NGR-GFP-ΔNBax fusion protein was added to HUVEC cells, the cell death ratio after 24 hours was 4 to more than 10 times higher than that of HeLa cells treated with NGR-GFP-ΔNBax fusion protein, where HeLa cells don't undergo angiogenesis,.

Table 1

| Experiment 1 | Cell Death (PI positive ratio) after 48 hours | | |
|---|---|---|---|
| | NGR-GFP-ΔNBax | Control (solvent only added) | No addition |
| HUVEC | 42.4% | 11.9% | 8.5% |

| Experiment 2 | Cell Death (PI positive ratio) after 24 hours | | |
|---|---|---|---|
| | NGR-GFP-ΔNBax | | No addition |
| | 200 ng | 60 ng | |
| HUVEC cell | 13.1% | 5.4% | 1.4% |
| HeLa cell | 3.2% | 0.4% | 0.4% |

Note: "No addition" means PI positive ratio of cells in the normal medium without any extra addition nor any extra treatment.

To confirm that RGD-GFP-ΔNBax protein and NGR-GFP-ΔNBax protein are introduced into the cells, $1 \times 10^5$ HUVEC cells and $5 \times 10^4$ HeLa cells were plated in each well of a 4-well plate (Sonic Seal Slide; LAB-TEK Co.). Two hundred ng of NGR-GFP-ΔNBax fusion protein was added to each well. After 3 hours, medium was changed and PI (5 μM) was added. Fluorescence of GFP and PI was observed using a Confocal Laser Scanning Microscope (FLUOVIEWFV300 OLYMPUS) (with 10x objective lens). Figure 2 shows the presence of GFP inside the cells. While HUVEC cells undergoing cell death were shown to emit the fluorescence of GFP inside the cells (the nuclei of dead cells appear red), no fluorescence was observed in HeLa cells, indicating that the fusion protein was incorporated into only HUVEC cells, which were the model of endothelial cells undergoing angiogenesis, by the action of a homing signal peptide, NGR. Figure 3 shows cells stained with PI. The left figure shows HUVEC cells treated with NGR-GFP-ΔNBax fusion protein, and the right figure shows HeLa cells treated with NGR-GFP-ΔNBax fusion protein. These figures indicate that the number of PIpositive HUVEC cells is higher than that of PI-positive HeLa cells. Below Figure 3, the enlarged view of HUVEC cells treated with NGR-GFP-ΔNBax protein inside the box in the left image

of Figure 3 is shown. In this enlarged view, the cell indicated with PI (PI positive cells) is dead and the cell indicated with H (Hoechst positive cells) is living.

[Example 5] Anti-tumor effect of NGR-GFP-ΔNBax on tumor-bearing mice.

Tumor-bearing mice were prepared by subcutaneously transplanting HeLa cells ($1 \times 10^7$ cells) to nude mice BALB/c-nu/nu Slc (female, 8 weeks old). The size of tumor (volume) was determined under anesthesia (Nembutal) by measuring the long and short diameters with a precision caliper and calculating using conventional equation (long diameter) × (short diameter)$^2$/2. NGR-GFP-ΔNBax was synthesized using RTSHY500 kit (Roche). The synthetic reaction mixture 100 μl was centrifuged (12,000 rpm, 4°C, 10 minutes). After removing the supernatant, the precipitant was re-dissolved in 100 μl of the dissolving solution (6M UREA, 0.15M NaCl, 20 mM Hepes pH7.2). After standing at room temperature for 10 min, the solution was centrifuged (12,000 rpm, 4°C, 10 minutes), and the supernatant was used as NGR-GFP-ΔNBax sample. The concentration of NGR-GFP-ΔNBax was determined to be 500 ng/μl by conventional SDS-PAGE followed by staining with coomassie brilliant blue (CBB) using bovine serum albumin of known concentration as the standard. NGR-GFP-ΔNBax 50 μl was injected twice directly to a tumor (0.2-0.4 $cm^3$) of the 3 tumor-bearing mice after the size of the tumors was measured under Nembutal anesthesia. As controls 3 tumor-bearing mice were injected with the dissolving solution (6M UREA, 0.15M NaCl, 20 mM Hepes pH7.2) only. After one week, the size of the tumor was measured (Figure 4, 1W; white circle, NGR-GFP-ΔNBax sample was administered; solid circle, control). Further, for each two mice, NGR-GFP-ΔNBax sample and the dissolving solution (6M UREA, 0.15M NaCl, 20 mM Hepes pH7.2) as control were injected twice each with 50 μl directly to the tumor in the same way as the first administration, and after one week the size of the tumors was measured (Figure 4, 2W). In the figure, the rate of volume increase of each tumor (white circle, NGR-GFP-ΔNBax sample is administered; solid circle, control) is expressed as the percentage (%) of the tumor volume before the first injection, and the average (horizontal bar) is also shown. In the measurement after 1 week of the first injection, the standard deviations were shown with horizontal bars, and the statistical analysis

by Student's t-test indicated statistical significance. The decrease of tumor volume by NGR-GFP-ΔNBax administration (once and twice) was observed.

Industrial Applicability

As shown in the results of Example 4, the fusion protein, in which the homing signal peptide, GFP and ΔNBax are fused in this order, induced cell death specifically and potently in cells undergoing angiogenesis. This suggests that the fusion protein is specifically incorporated into cells undergoing angiogenesis by the action of the homing signal peptide in the fusion protein, and the cell death is induced by the action of ΔNBax in which the cell death-inducing activity is enhanced by GFP. Further, as shown in Example 5, the administration of the fusion protein, in which the homing signal peptide, GFP and ΔNBax are fused in this order, to tumor bearing mice caused the decrease in tumor volume. These results clearly indicate that the fusion protein of the present invention can induce, specifically and potently, the cell death of cancer cells undergoing angiogenesis and is useful as an inhibitor of cancer cell growth, that is an anti-cancer drug.

Table of sequences free text
SEQ ID NO: 7-18: homing signal peptides
SEQ ID NO: 19-27: primers

The whole contents of all the publications referred herein are hereby incorporated. It is to be understood that variations and modifications may be possible within the purview and the cope described in attached claims by those skilled in the art. It is intended that the present invention includes such variations and modifications.